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Original article

Chronic Cellular Hyperexcitability in Elderly Epileptic Rats with Spontaneous Seizures Induced by Kainic Acid Status Epilepticus while Young Adults

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ABSTRACT: Emerging data indicate that age-related brain changes alter seizure susceptibility, seizure-associated neurodegeneration, and responsiveness to AEDs. The present study assessed long-term animal survival in the Kainic Acid (KA) model along with *in-vivo* spontaneous seizure frequency, cellular hyperexcitability in CA1 *in-vitro* and *in-vivo* in subiculum, and responsiveness of *in-vitro* CA1 hyperexcitability to topiramate. Sprague-Dawley male rats were given KA to induce convulsive status epilepticus (KA-SE) at 2-3 months of age. The one-month mortality after KA-SE was 27%. One-month survivor rats had 37% sudden unexplained late mortality after KA-SE as compared to none in saline controls during their second year of life. *In-vivo* seizure frequency was examined prior to terminal experiments. The diurnal average seizure frequency in the KA-SE group at age 2 years was 1.06 ± 0.24 seizures/hour while no seizures were observed in the saline age-matched controls (p<0.001). In-vitro recordings of CA1 pyramidal neurons revealed that depolarizing current injection from -60 mV evoked an increased number of action potentials in the aged KA-SE group compared to controls (p<0.002). Topiramate exhibited dose-dependent inhibition of action potential firing evoked by current injections into CA1 pyramidal neurons of KA-SE rats. In subiculum, KA-SE rats had frequent interictal spikes associated with high frequency oscillations while only rare spontaneous EPSPs were recorded in saline controls. Our experiments revealed that the hippocampal formation of aged epileptic rats shares features of hyperexcitability previously described in young adult epileptic rats using the KA model.

Key words: Topiramate; Aged; Epilepsy; Bursting; Hippocampal slice; SUDEP

There are limited studies of seizures and epilepsy in older animals. Most studies in elderly models have examined acute provoked seizures in otherwise "normal" aged rodents to examine age-related changes in seizure thresholds or responsiveness to anti-epileptic drugs [1,2]. Similarly to young adult rodents, aged rodents are susceptible to seizures when exposed to chemoconvulsants such as pentylenetetrazol, Kainic Acid (KA), or pilocarpine, but most studies suggest that the aged rodent brain has a higher threshold to evoke a convulsive seizure [3,4]. Despite this apparent higher seizure threshold, the aged rodent brain exhibits a greater amount of neurodegeneration after KA [5-8]. It is also noteworthy that seizures induced in the aged brain are inhibited by lower doses of GABAergic AEDs [1]. Thus, emerging data indicate that age-related brain changes can alter seizure susceptibility, seizure-

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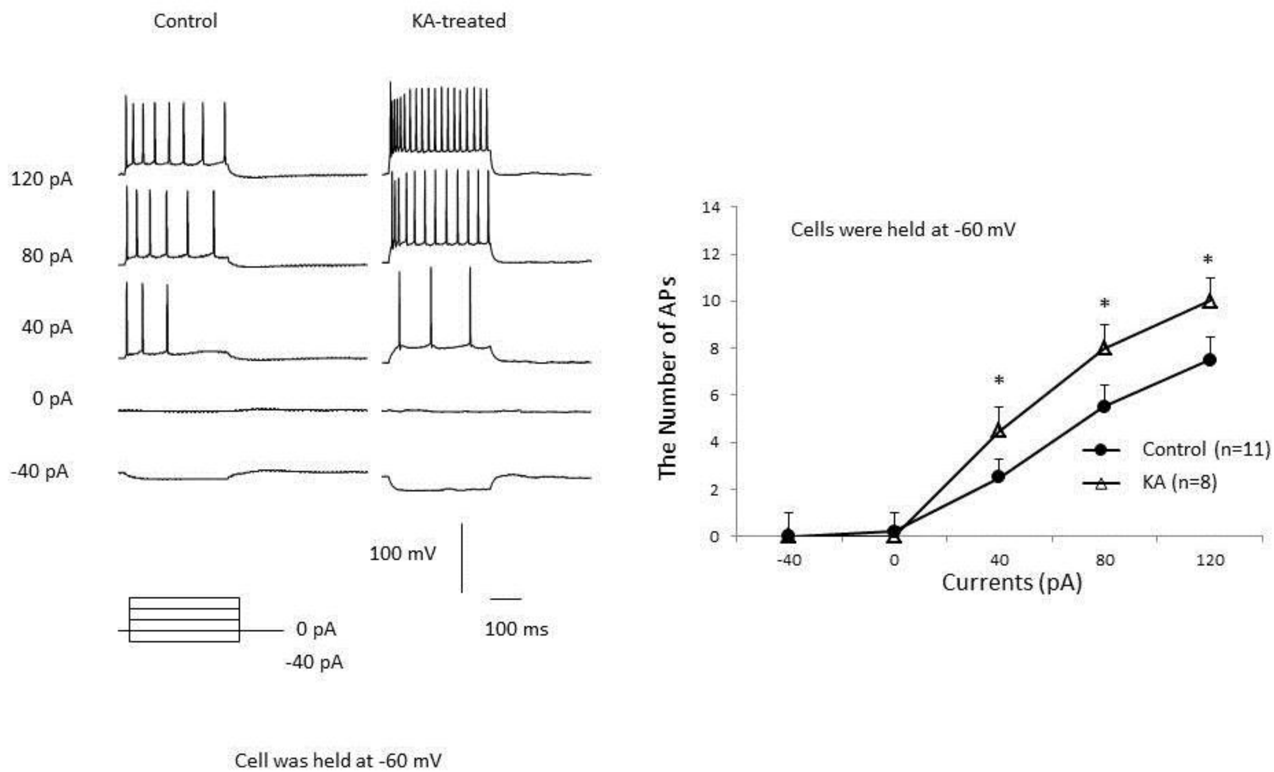

Figure 1. CA1 cellular hyperexcitability in aged KA rats as compared to aged controls. Left, depolarizing currents (-40, 0, 40, 80, 120 pAs) evoked significant more APs in hippocampal CA1 pyramidal neurons from a KA-treated rat compared to the number of APs evoked in a neuron from a Control rat. Right, pooled data shows that there is significant increase in the number of APs evoked by incremental steps of currents in KA-treated group compared with the Control when held at -60 mV ($p<0.002$, Two-way ANOVA, Control, n=11, KA, n=8).

associated neurodegeneration, and responsiveness to AEDs.

While the effects of aging *per se* on seizures and epilepsy are beginning to emerge, a yet unaddressed question is whether these differ in the aged brain that has experienced spontaneous recurrent seizures in young adulthood, as there are no studies that have focused on aged animals after an extended chronic epileptic state. The current study examined *in-vivo* seizure frequency, *in-vitro* hippocampal neuronal activity in the CA1 region, *in-vivo* neuronal activity in the subiculum, and *in-vitro* responses to anticonvulsant treatment in 2-year-old rats that experienced kainic acid induced status epilepticus (KA-SE) at 2-3 months of age and later became epileptic with spontaneous partial seizures. The results suggest that overt features of the chronically epileptic aged brain do not differ strikingly from the younger adult epileptic brain.

MATERIALS AND METHODS

Male Sprague Dawley rats (Harlan) 250-350 grams (age 60 – 90 days) were used for these experiments, and were received as adults in groups of 8-10 rats at a time. Rats were housed individually at the VA animal facility on a 12-h light/dark cycle with water and food ad libitum. Lights were turn-on at 7 am, and experiments were typically started at 9 am. All procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with NIH guidelines on the ethical use of experimental animals.

Systemic kainic acid model

Sprague-Dawley rats receive repeated subcutaneous injections of 2.5 mg/kg of kainic acid (OPIKA-1, Ocean Produce Inc., Shelburne, NS) in 0.9% saline (pH 7.4) at least one week after they were received. Three to four injections of 2.5 mg/kg of KA were given every 30 or 45 minutes until the animals developed motor seizures. Rats were monitored for behavioral signs of seizures for at least 5 h after the first injection and were given a maximum seizure severity score every 15 min interval using a previously validated scale [9, 10]. The maximum score during the 15 min span was considered the seizure score for that period, and the scores were added for the entire period of observation to assess severity of the convulsive status epilepticus. Convulsive status epilepticus was defined as two consecutive 15 min periods with motor seizures. Motor seizures were defined according to a modified Racine's scale with motor seizures indicating at least unilateral (level 3) or bilateral (level 4) clonic forelimb activity resulting in rearing and falling (level 5) [11]. If no consecutive periods with motor seizures were recorded, those rats were excluded from the analysis (2 out of 32), as convulsive status epilepticus implies continuous convulsive motor seizures for at least 30 minutes. The sum of the scores for each 15-minute period during the 5 hours of observations after the Kainic Acid injection was considered as severity of KA-induced status epilepticus (KA-SE). Rats did not receive benzodiazepines or other anticonvulsant treatment. The experiments were performed in batches of 8-10 age-matched controls, typically with 3-4 rats receiving three injections of vehicle (0.9% sodium chloride - saline) every 45 min, while the other 5-6 rats received KA. The acute mortality rate was defined as death within one month from KA-SE or saline injections, typically within the first week after KA. Late mortality was defined as death between the one-month survival after KA-SE or saline until just prior to the electrode implantation or terminal experiment at 2 years of age. The rats were housed individually at the animal care facility until they were about 2 years of age, when they were monitored for spontaneous seizures [12]. The late development of chronic spontaneous seizures was verified by EEG and behavioral observation at 20-22 months after kainic acid injections and within 2 weeks from in-vitro or in-vivo terminal experiments. Animals were visited daily, often observed to experience motor seizures (level 3 or higher in Racine's scale), and appeared more aggressive as compared to their saline treated age-matched controls.

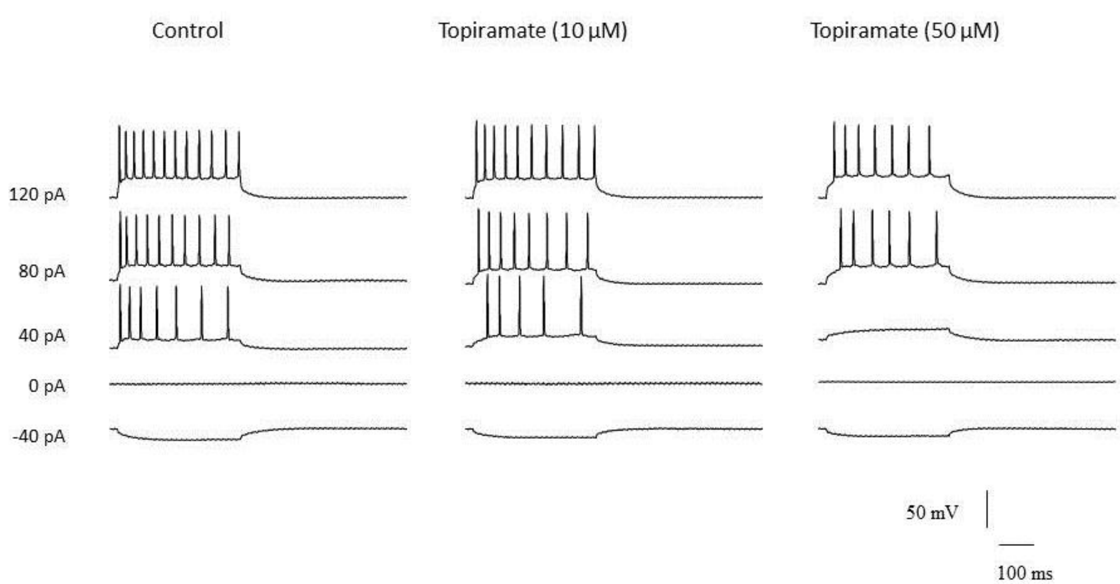

Figure 2. Topiramate diminishes CA1 hyperexcitability in aged rats in dose dependent manner. A representative recording shows that 10 µM (middle) and 50 µM (right) topiramate inhibited AP generation induced by depolarizing currents.

Electrode implantation

Rats were anesthetized by injection of pentobarbital (50 mg/kg ip) or ketamine (57 mg/kg ip) and placed on a stereotactic apparatus. A pair of insulated stainless steel twisted bipolar electrodes (tips were separated by 3 mm) were implanted into the hippocampus according to the following coordinates: 3.9 mm posterior (AP), 1.7 mm lateral, and 3.9 mm ventral from bregma. Four stainless steel screws were implanted in the skull, and one was used as the ground electrode. The implant was anchored to dental cement that was added to secure the electrode to the skull. Animals were given acetaminophen (100 mg/kg oral) after surgery and were allowed to recover for one week prior to EEG recordings. The opposite hippocampus was used for *in-vivo* or *in-vitro* experiments within 2-3 weeks after the EEG recordings that verified seizure frequency. All animals (implanted or not) that had experienced sustained KA-SE as defined as two consecutive periods of convulsive motor seizures demonstrated spontaneous behavioral seizures at some point of observation within last 6 months prior to experiments at 2 years. All 16 of the KA-SE rats that had survived were implanted and demonstrated behavioral and electroencephalographic seizures.

In-vivo EEG recording

EEG recordings from implanted electrodes were recorded with a CyberAmp amplifier (Molecular Device, Inc.). The signals were filtered using a low frequency filter (LFF) of 1 Hz and a high frequency filter (HFF) of 60 Hz. They were converted from analog to digital by sampling at 500 Hz using a Digidata 1322 (Molecular Devices, Inc.) for acquisition to computer. An average of 7.6 hours of EEG per day was recorded for each rat starting at 9 am for at least two consecutive days until 5 spontaneous seizures were recorded or five periods of 7.6 hours were recorded. The reason of the arbitrary amount of EEG recording (7.6) is due to the maximum capacity of data recorded using a DVD+R format in our recording system.

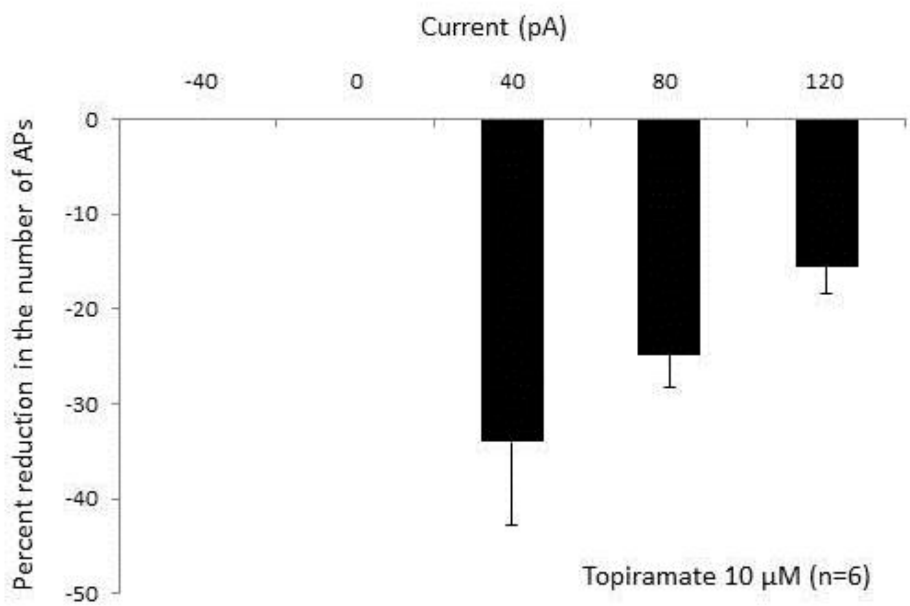

Figure 3. Topiramate diminishes CA1 hyperexcitability in aged KA rats . The percentage of reduction in the number of evoked APs from the pre-treated level by 10 µM topiramate in each current dose. The pooled data was generated from 6 out of 12 neurons from KA-treated rats that were treated with bath-applied 10 µM topiramate.

Hippocampal slice preparation

Rats were decapitated and the brains were immediately dissected and placed in ice-cold artificial cerebrospinal fluid (aCSF) that contained (in mM): sucrose 201, KCL 3.2, NaH2PO4 1.25, MgCl2 2, CaCl2 2, NaHCO3 26, and D-glucose 10; the aCSF solution was bubbled with 95% O_2 / 5% CO_2. After one hour, the brains were cut and glued to the stage of a vibratome (Leica 1000s, Leica Microsystems, Wetzlar, Germany) bathed by ice-cold aCSF. Coronal sections (370 µm) of slices were cut from the hippocampus opposite to the implanted electrode, continuously oxygenated in sucrose aCSF, and then incubated for ≥1 h in a custom-made holding chamber filled with continuously oxygenated recording aCSF that contained (in mM): NaCl 126, KCL 3.3, NaH2PO4 1.25, MgSo4 1.3, CaCl2 2, NaHCO3 26, and D-glucose 10 bubbled with 95%/5%CO2 at room temperature.

In vitro electrophysiological experiments

Hippocampal slices were transferred to a submersion chamber that was perfused continuously with oxygenated aCSF at room temperature for recordings. Whole-cell patch-clamp recordings were obtained from CA1 pyramidal neurons under visual guidance using infrared differential interference contrast microscopy (Zeiss Axioskop FS2, Zeiss, oberkochen, Germany with a Dage-MTI camera, USA). The pipette solution contained (in mM): K-gluconate 130, NaCl 1, MgCl2 1, CaCl2 1, EGTA 5, HEPES 10, KOH 3, Mg-ATP 4 (pH 7.2). The CA1b pyramidal cell layer was targeted with micropipettes that were prepared using a Flaming-Brown P-97 puller (Sutter Instruments, USA). The filled recording pipettes had resistances of 3-5 MΩ, and series resistance were <20 MΩ. Current-clamp recordings were obtained by using an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, U.S.A.) and digitized with a Digidata 1322A (Molecular Devices, Sunnyvale, CA, U.S.A.) for acquisition to computer. Current-clamp protocols were generated, and data were acquired to computer using PClamp 7. Data were low-pass filtered at 2 KHz and digitized at 10 KHz. In current-clamp studies, cells were held at -60 mV while a series of current steps (-40 to 120 pA in 40 pA increments for 600 ms) were injected.

In vivo electrophysiological experiments

Sprague Dawley male rats two years after saline or KA-SE (3 in each group) were also used for in vivo experiments. Recordings of spontaneous activity in the subiculum were obtained under urethane anesthesia (1.5 mg/kg), with tungsten recording electrodes (0.5MΩ) inserted 6.3mm posterior from bregma and 2.5mm lateral from midline. Depth of recording was 4.5mm from the dorsal surface of the brain. Spontaneous fEPSPs were seen in the subiculum of both groups of rats, but they were very rare in controls (~ 1 in several minutes).

Statistical analysis

The severity of epilepsy was assessed by counting the hourly frequency of spontaneous seizures about 1 – 2 weeks prior to the slice or in-vivo experiments. Seizure frequency was expressed as mean ± SEM during a 7.6 hour recording. The two groups (KA-SE and Saline) were compared using standard *t*-test with unequal variance. For the intracellular experiments, the number of action potentials evoked at each current step was counted and the group data are presented as mean ± SEM. Two-way analysis of variance (ANOVA) was used for comparison of the number of evoked action potentials between the control and KA-SE groups across the step series of current injections. A $p < 0.05$ was considered significantly different.

RESULTS

The acute mortality as defined as within one-month after KA-SE or saline injections was 27% (n=8/30) and 0 % respectively. There were 8 additional KA rats who had been observed to have spontaneous motor seizures who were found dead in their cages (as their water and food were filled in the morning) between ages 11 and 24 months. This is 37% late mortality (n= 8/22) for a cohort of one-month survivors after KA-SE. The presumed cause of death was sudden unexpected death in epilepsy (SUDEP) as rats had foaming in the month and no other gross abnormality. One saline rat was euthanized after developing a large soft tissue tumor over its neck at age 16 months (n= 1/20) but no saline injected rats died suddenly and unexplained as the KA-SE group.

KA-treated rats that experienced status epilepticus at age 2-3 months had spontaneous non-convulsive seizures with a frequency at age 2 years of 1.06 ± 0.24 seizures per hour (n = 16) as compared to none seizures in the saline treated group that was recorded (n = 14; p<0.001). Most of the recorded seizures (92%) were complex partial seizures (Racine level 1 or 2) with duration between 25 to 60 seconds.

CA1 pyramidal neurons were recorded in the KA-SE and Saline groups. There were no significant differences in intrinsic membrane properties such as resting membrane potential, input resistance, and capacitance. However, depolarizing current injection from -60 mV evoked an increased number of action potentials in the KA-treated group compared to the control group (p<0.002, n=10 control, n=8 KA; Figure 1), indicating increased neuronal excitability in the KA-treated rats.

In 12 CA1 pyramidal neurons from 10 KA rats, we examined the effect of topiramate (10 and 50 µM) on the responses to depolarizing current injection. Topiramate inhibited the numbers of action potentials in response to depolarizing current injection in 6 out of 12 neurons tested. Figure 2 shows raw voltage responses for a CA1 pyramidal neuron from a KA-treated rat that exhibited dose-dependent inhibition of action potential firing. Pooled summary data for the six cells that responded to 10 µM topiramate are shown in Figure 3. In 5 out of 6 CA1 pyramidal neurons, we observed an increased effect on inhibition of action potential firing with 50 µM topiramate.

In vivo recordings under urethrane anesthesia were obtained from the subiculum in KA-SE and saline-treated rats at age 2 years. As shown in Figure 4, recordings from KA-treated rats revealed frequent interictal spikes associated with high frequency oscillations (i.e., fast ripples). Very rare field EPSPs were observed in saline-treated age-matched control rats, but they did not have a high frequency oscillation component.

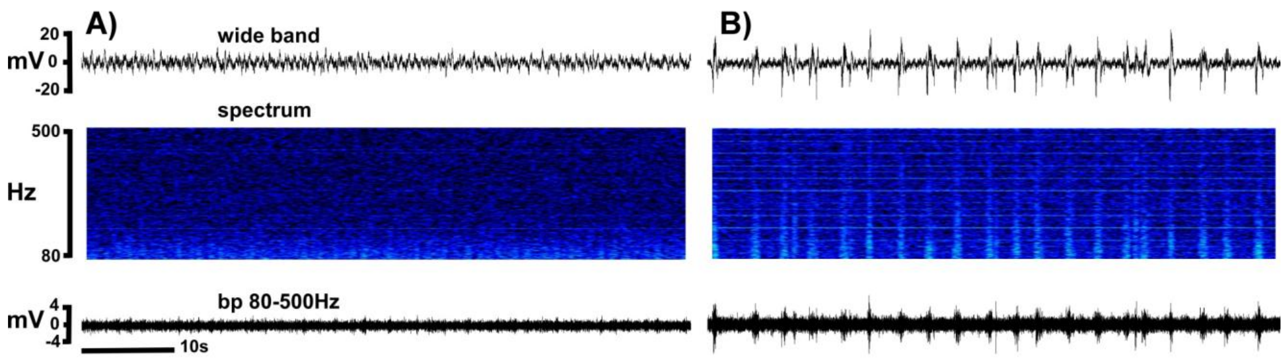

Figure 4. **Spontaneous interictal spikes with fast ripples in Subiculum of aged KA rats but not aged controls.** Spontaneous activity recorded in the subiculum of 2 year old rats who were exposed to Saline (left column) or experienced Kainic Acid status epilepticus (right column) at 3 months of age. The compressed timeline shows the frequency of spontaneous interictal discharges in the KA but not in the control group. The top recording shows a representative 1 minute trace with open filters, while the bottom recording has a filtered wide-band between 80 and 500Hz from the same trace. The middle graph is a power spectrum representation of the same record (with filters: LF – 80 Hz and HF – 500 Hz) to demonstrate the presence of high frequency activity associated with the interictal discharges. High frequency oscillations (or fast ripples) are often associated with interictal discharges near the seizure onset zone in the Kainic acid model.

DISCUSSION

Our study revealed that: a) Spontaneous seizures were recorded at a stable frequency 20-22 months after KA-induced status epilepticus and not in saline-treated controls; b) A substantial number of KA rats who had behavioral motor seizures died unexplained prior to 2 years of age as compared none of the saline treated controls; c) Spontaneous interictal spikes indicative of cellular hyperexcitability were recorded at 2 years of age in the subiculum of aged rats that were treated with KA at age 2-3 months but not in saline treated controls; d) Current injection in CA1 pyramidal neurons induced a greater number of action potentials in slices obtained from 2 year old rats who had experience KA induced status epilepticus at 2-3 months of age as compared to saline-treated age matched controls; and e) Topiramate induced a dose-dependent decrease in the numbers of action potentials induced by current injection in CA1 pyramidal neurons obtained from 2 year old rats who had KA-SE or were saline treated at age 2-3 months.

Although we did not compare aged to young adult rats, our data indicated that the phenomenology of seizures and hippocampal excitability in aged rats that acquired epilepsy in young adulthood did not differ from that previously observed for epileptic animals in early adulthood. Spontaneous seizure frequency at nearly 2 years after repeated low-dose KA treatment was not dramatically different from that reported 3-4 months after KA in young adult rats [12]. Furthermore, intrinsic hyper-excitability was observed in surviving CA1 pyramidal neurons, as manifest by increased evoked action potential firing and Subicular neurons as demonstrated by frequent interictal discharges.

Inhibition of pyramidal neuron action potential firing was observed by topiramate at concentrations previously shown to inhibit voltage-gated sodium channels and epileptiform activity in vitro [13, 14]. Notably, this was observed in only 50% of neurons tested, and this could have reflected age-associated differences in voltage-gated sodium or potassium channel function, as topiramate can act on each of these [14-18]. However, given the relative paucity of pharmacological studies of topiramate in the aged brain, it is not possible to determine if the limited in vitro efficacy of topiramate in the current study resulted from age-associated neuronal changes or changes that resulted from the prolonged period of spontaneous recurrent seizures in this experimental group. Additionally, it remains unknown whether this limited efficacy in vitro could translate to limited efficacy in vivo given the multiple potential mechanisms of action of topiramate beyond voltage-gated ion channel modulation.

Overall, our data revealed that the hippocampal formation of aged epileptic rats shares features of hyper-excitability previously described in young adult epileptic rats using the KA model. Further study may identify more subtle differences between the aged and younger adult epileptic brain, but also may reveal that the age of initial seizure onset could be a more critical factor in the disease semiology within the elderly population.

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